Role of Phosphate Transport System Component PstB1 in Phosphate Internalization by *Nostoc punctiforme*

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ABSTRACT

In bacteria, limited phosphate availability promotes the synthesis of active uptake systems, such as the Pst phosphate transport system. To understand the mechanisms that facilitate phosphate accumulation in the cyanobacterium *Nostoc punctiforme*, phosphate transport systems were identified, revealing a redundancy of Pst phosphate uptake systems that exists across three distinct operons. Four separate PstB system components were identified. *pstB1* was determined to be a suitable target for creating phenotypic mutations that could result in the accumulation of excessive levels of phosphate through its overexpression or in a reduction of the capacity to accumulate phosphate through its deletion. Using quantitative real-time PCR (qPCR), it was determined that *pstB1* mRNA levels increased significantly over 64 h in cells cultured in 0 mM added phosphate and decreased significantly in cells exposed to high (12.8 mM) phosphate concentrations compared to the level in cells cultured under normal (0.8 mM) conditions. Possible compensation for the loss of PstB1 was observed when *pstB2*, *pstB3*, and *pstB4* mRNA levels increased, particularly in cells starved of phosphate. The overexpression of *pstB1* increased phosphate uptake by *N. punctiforme* and was shown to functionally complement the loss of PstB in *E. coli* PstB knockout (PstB[−]) mutants. The knockout of *pstB1* in *N. punctiforme* did not have a significant effect on cellular phosphate accumulation or growth for the most part, which is attributed to the compensation for the loss of PstB1 by alterations in the *pstB2*, *pstB3*, and *pstB4* mRNA levels. This study provides novel *in vivo* evidence that PstB1 plays a functional role in phosphate uptake in *N. punctiforme*.

IMPORTANCE

Cyanobacteria have been evolving over 3.5 billion years and have become highly adept at growing under limiting nutrient levels. Phosphate is crucial for the survival and prosperity of all organisms. In bacteria, limited phosphate availability promotes the synthesis of active uptake systems. The Pst phosphate transport system is one such system, responsible for the internalization of phosphate when cells are in phosphate-limited environments. Our investigations reveal the presence of multiple Pst phosphate uptake systems that exist across three distinct operons in *Nostoc punctiforme* and functionally characterize the role of the gene product PstB1 as being crucial for the maintenance of phosphate accumulation. We demonstrate that the genes *pstB2*, *pstB3*, and *pstB4* show alterations in expression to compensate for the deletion of *pstB1*. The overall outcomes of this work provide insights as to the complex transport mechanisms that exist in cyanobacteria like *N. punctiforme*, allowing them to thrive in low-phosphate environments.

Phosphorus (P) is a crucial element for the survival and growth of all organisms, and it is commonly associated with oxygen in the form of inorganic phosphates (PO₄[−] and P_i) in the environment. Phosphate has a central role in many of the metabolic and energy-producing pathways in bacteria. For example, diazotrophs like cyanobacteria are unable to fix atmospheric nitrogen in low-phosphate environments (1). A low abundance of P_i is typical of soils after intensive plant cropping, and even if abundant, P_i is often complexed to soil constituents, making it unavailable to many organisms in this environment (2). In aquatic systems, P_i is relatively less soluble than in soils, making it a key growth-limiting factor (3). Thus, the survival of an organism within a low-P_i environment can be linked to the evolution of mechanisms that increase the efficiency of P_i uptake and retention.

Cyanobacteria such as *Nostoc punctiforme* have evolved mechanisms that make them adept at acquiring and storing nutrients that are at low extracellular concentrations, including phosphorus in P_i-limited environments (4). Through understanding the P_i acquisition mechanisms of cyanobacteria, we aim to characterize the genetic mechanisms involved in P_i uptake. *Nostoc punctiforme* exhibits extensive phenotypes, including photoautotrophic, fac-

ultative heterotrophic, and diazotrophic modes. It is globally distributed, inhabits fresh water and soil environments, and grows as both a free-living organism and in symbiotic association with a broad range of hosts, including fungi, plants, and lichens (5).

Many cyanobacterial species exhibit various mechanisms for P_i uptake in P_i-limiting and P_i-saturated environments. The scavenging of P_i by the unicellular marine cyanobacterium *Crocosphera watsonii* is due to high-affinity P_i transportation systems, and this key adaptation allows survival under low-P_i conditions (6).

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FIG 1 Overview of the Pst phosphate transport system (45–47). The Pst system is comprised of PstS, PstC, PstA, and PstB. PstS is the periplasmic P_i -binding protein, PstA and PstC form the membrane channel, PstB is membrane bound and is the ATP hydrolysis site, PhoU is a regulatory protein that interacts with PstB and PhoB to regulate the import of P_i through the Pst system, and PhoR is the P_i -sensing/-regulatory site (45–47).

For the model cyanobacterium *N. punctiforme*, little is known about its P_i transportation mechanisms; however, 262 open reading frames (ORFs) are predicted to encode proteins that transport organic and inorganic molecules across the cell membrane, and of these, 89 ORFs encode ABC (ATP binding cassette) transporters, to which the Pst transport system belongs (5).

Two major bacterial P_i import systems exist in bacteria, the Pst and Pit systems. In *Escherichia coli*, P_i is transported via the Pit system when the external phosphate concentration is lower than 20 μM , and it is at this concentration that the Pst system is induced (7). The Pit system is comprised of two systems, called PitA and PitB (8). PitA is expressed constitutively, is dependent on the proton motive force within the periplasmic space of the cell, and has a 100-times-greater affinity for P_i than the Pst system, and yet, both the Pit and Pst systems transport P_i at the same rate of 70 $\text{nmol} \cdot \text{min}^{-1}$ (7). *pitB* encodes a functional P_i transporter that may be repressed at low P_i levels by the *pho* regulon, and it is assumed that this secondary system is linked to low-phosphate environments (8).

The Pst system is highly efficient at transporting P_i , particularly in cells growing under low- P_i concentrations, where it is capable of transporting higher levels of P_i overall than the Pit system (7). The Pst system is encoded by several genes that make up the *pho* regulon and is essential in the regulation of P_i internalization. The Pst system is typically comprised of one periplasmic substrate binding protein (PstS), two membrane-bound components (PstA and PstC), and the cytosolic PstB, which energizes the release of free P_i in the cytoplasm (Fig. 1). In *E. coli*, *pst* genes, along with the *phoU* gene, form the *pstSCAB-phoU* operon (Fig. 2A) (7).

In the periplasm, complex P_i is hydrolyzed via alkaline phosphatase (7), and it has been shown that within the periplasm of *E. coli*, alkaline phosphatase and solute binding proteins allow *E. coli* to metabolize many nontransportable P_i esters, thus increasing the bioavailability of environmental P_i sources (9). The Pst system is anchored into the inner cell membrane by two multimembrane-spanning proteins, PstC and PstA, to form a transmembrane

(TM) channel that transports P_i through the cytoplasmic membrane (7). The *pstA* gene encodes an integral membrane protein with six transmembrane helices (7), and its disruption reduces P_i transport, most likely by reducing the affinity of the protein and, hence, the Pst system, for insertion into the membrane. In addition, mutations in the *pstC* gene affect the ability for P_i transport to occur without affecting the regulation of *phoA*, which is considered the controller of the P_i uptake rate. The cytoplasmic sides of PstC and PstA are linked to the Pst system component PstB, which is involved in the ATP hydrolysis needed for energizing the TM channel, allowing it to function as a P_i transporter (7). It is predicted that mutations of the *pstB* gene would affect the internalization of P_i in cells and have a negative effect on *phoA* expression (7).

Two copies of Pst systems, known as Pst1 and Pst2, have been identified in the genome of the freshwater cyanobacterium *Synechocystis* sp. strain PCC 6803 (10). Both Pst systems encode ABC transporters and show upregulation under P_i -limiting conditions (10). The two Pst systems of *Synechocystis* sp. strain PCC 6803 are differentially expressed and dependent upon extracellular P_i levels (11). The transport of P_i is primarily dependent on Pst1, since the contribution of Pst2 typically occurs only under low P_i levels (10).

It is predicted that 50% of the freshwater cyanobacterial strains contain duplicate Pst systems (12); however, to date, no evidence of gene expression or characterization of function exists for many of these systems (13). The Pst1 system of *Synechocystis* sp. strain PCC 6803 is generally considered to be a low- or medium-affinity P_i transporter, as this organism inhabits freshwater environments where P_i levels are not typically limiting, and this is the converse of the Pst1 system of *E. coli*.

Basic Local Alignment Search Tool (BLAST) and sequence identity analyses using established PstB primary peptide data from *E. coli*, *Bacillus subtilis*, *C. watsonii*, and *Synechocystis* sp. strain PCC 6803 revealed the presence of four homologous sequences in the genome of *N. punctiforme* (Table 1 and Fig. 2B to D). The inferred PstB1, PstB2, PstB3, and PstB4 peptide sequences of *N. punctiforme* shared identities of 59, 50, 57, and 54%, respectively, with the PstB of *B. subtilis*, 60, 46, 52, and 52%, respectively, with the PstB of *E. coli*, 65, 46, 53, and 60%, respectively, with the PstB of *Synechocystis* sp. strain PCC 6803, and 67, 45, 52, and 56%, respectively, with the PstB of *C. watsonii* (see Fig. S1 in the supplemental material). The sequence identities between PstB1 and PstB2, PstB3, and PstB4 were 47, 57, and 60%, respectively. PstB1 (Npun_F0894) was selected for further characterization, as it is the most similar to the previously characterized PstBs of *B. subtilis*, *E. coli*, *Synechocystis* sp. strain PCC 6803, and *C. watsonii* and would therefore potentially have a comparable function and most likely generate phenotypes of altered capacity to accumulate phosphate when deleted by insertional mutagenesis or overexpressed.

MATERIALS AND METHODS

***Nostoc punctiforme* strains and culturing conditions.** *Nostoc punctiforme* PCC 73120 maintenance cultures were grown in 500 ml of Allen and Arnon broth as described previously (14), in 2-liter conical flasks covered with sterile aluminum foil. All cultures were incubated at 25°C, with 16-h-light (cool white fluorescent light at 70 $\mu\text{mol}/\text{m}^2/\text{s}$) and 8-h-dark cycling and constant agitation on a rotary shaker (160 rpm). Cells were harvested by centrifugation (1,500 $\times g$ for 10 min) and resuspended in fresh medium every 2 weeks. All culture manipulations were performed under aseptic conditions. For all P_i treatments, *N. punctiforme* cells ($\sim 1,000$ mg wet weight) were pelleted and washed five times in sterile

FIG 2 Overview of the *pst* transport systems and their potential regulatory genes in *E. coli* (A) and *N. punctiforme* (B to E). (A) Phosphate import ATP-binding protein gene *pstB* (GenBank accession no. [BAJ45469](#)), upstream phosphate ABC transporter periplasmic substrate-binding protein gene *pstS* (accession no. [BAJ45472](#)), upstream phosphate ABC-type transport system permease component gene *pstC* (accession no. [BAJ45471](#)), upstream phosphate transporter permease subunit gene *pstA* (accession no. [BAJ45470](#)), downstream transcriptional regulator gene *phoU* (accession no. [BAJ45468](#)), and downstream transcriptional antiterminator gene *bglG* (accession no. [BAJ45467](#)). (B) Putative phosphate ABC transporter gene *pstB1* (accession no. [WP_012407653.1](#), Pfam no. PF0005), upstream putative phosphate ABC transporter inner membrane subunit gene *pstA* (accession no. [WP_012407652.1](#), Pfam no. PF00528), upstream putative phosphate ABC transporter periplasmic phosphate binding protein gene *pstS* (accession no. [WP_012407650.1](#), Pfam no. PF12849), upstream putative biotin uptake component gene *bioY* (accession no. [WP_012407649.1](#), Pfam no. PF02632), upstream putative lipoprotein signal peptidase *ispA* (accession no. [WP_012407648.1](#), Pfam no. PF01252), downstream putative glycine tRNA ligase gene (black arrow), downstream putative hypothetical protein gene (accession no. [WP_012407657.1](#)) (gray arrow), and downstream GAF sensor hybrid histidine kinase gene (accession no. [WP_012407655.1](#), Pfam no. PF01590). (C) Overview of *pstB2* (accession no. [WP_012410452.1](#), Pfam no. PF00005) and its surrounding genes, including downstream putative phosphate transport ATP binding protein gene *pstB3* (accession no. [WP_012410553.1](#), Pfam no. PF00005), downstream putative phosphate transport system permease protein gene *pstA* (accession no. [WP_012410554.1](#), Pfam no. PF00528), downstream putative phosphate transport system permease protein gene *pstC* (accession no. [WP_012410555.1](#), Pfam no. PF00528), upstream hypothetical protein gene (accession no. [WP_012410451.1](#)) (black arrow), upstream putative short-chain dehydrogenase (*adh_short*, accession no. [WP_012410450.1](#), Pfam no. PF00106), and upstream putative 4-hydroxyphenylpyruvate dioxygenase gene *cppA* (accession no. [WP_012410449.1](#), Pfam no. PF00903). (D) Overview of *pstB4* (accession no. [WP_012411066.1](#), Pfam no. PF00005) and its surrounding genes, including downstream putative phosphate transport system permease protein gene *pstA* (accession no. [WP_012411067.1](#), Pfam no. PF00528), downstream phosphate transport system permease protein gene *pstC* (accession no. [WP_012411068.1](#), Pfam no. PF00005), downstream putative periplasmic phosphate binding protein gene *pstS* (accession no. [WP_012411069.1](#), Pfam no. PF12849), downstream putative periplasmic sulfate binding protein gene *cysP* (accession no. [WP_012411070.1](#), Pfam no. PF13531), upstream hypothetical protein gene (accession no. [WP_012411065.1](#)) (left-facing black arrow), upstream putative single-stranded-DNA-specific exonuclease gene *recJ* (accession no. [WP_012411064.1](#), Pfam no. PF01368), and upstream hypothetical protein gene (accession no. [WP_012411063.1](#)) (right-facing black arrow). (E) Overview of potential *pst* regulatory elements *phoU* (accession no. [WP_012410558.1](#), Pfam no. PF01895), *phoB* (accession no. [WP_012410557.1](#), Pfam no. PF00512), and *phoR* (accession no. [WP_012410556.1](#), Pfam no. PF00072) and surrounding genes, including upstream hypothetical protein gene (accession no. [WP_012410555.1](#)) (left-facing black arrow), downstream hypothetical protein gene (accession no. [WP_012410559.1](#)) (right-facing black arrow), and downstream putative GtrA family protein gene *gtrA* (accession no. [WP_012410560.1](#), Pfam no. PF04138).

double-distilled water (ddH₂O) and then resuspended in 250 ml modified Allen and Arnon solution containing no added P_i (KH₂PO₄) and purged for 72 h. Cells were then pelleted, and 25 mg (wet weight) was resuspended in 100 ml of Allen and Arnon (~1 × 10⁸ cells/ml final density) containing the desired final P_i concentration, unless otherwise specified.

Escherichia coli strains and culturing conditions. *Escherichia coli* strain JW3703-1 Δ *pstB756::kan* (PstB knockout [PstB⁻] strain) (15) and the K-12 wild-type (WT) strain were obtained from the Coli Genetic Stock

Centre (CGSC, New Haven, CT, USA). For routine culturing of *E. coli*, Luria-Bertani (LB) liquid growth medium was used (16). For solid medium, the addition of 1.5% purified agar to the liquid LB was used as described previously (16). Incubation of *E. coli* was at a constant temperature of 37°C, and liquid cultures were shaken at 220 rpm on an orbital shaker. The growth of *E. coli* was measured using a spectrometer (BMG Labtech POLARstar OMEGA) to quantify the optical density based on the absorbance (optical density at 600 nm [OD₆₀₀]), where an OD₆₀₀ of 0.5

TABLE 1 Putative *pstB* genes in *N. punctiforme*

Gene	COG	Locus tag	NCBI accession no.	Size (bp)	DNA coordinates	Km ^r insert coordinate
<i>pstB1</i>	COG1117	Npun_F0894	WP_012407653.1	807	1054005–1054811	21054065
<i>pstB2</i>	COG1117	Npun_R4107	WP_012410452.1	780	5155866–5156645	NA ^a
<i>pstB3</i>	COG1117	Npun_R4108	WP_012410453.1	780	5157199–5157978	NA
<i>pstB4</i>	COG1117	Npun_R4757	WP_012411066.1	831	5896234–5897064	NA

^a NA, not applicable.

corresponds to the mid-exponential growth phase (16). The antibiotics ampicillin (100 µg/ml) and kanamycin (25 µg/ml) were added where appropriate. For all P_i treatments, *E. coli* cells at mid-exponential growth (OD₆₀₀ of 0.5) were pelleted and washed five times in TG medium (1% Bacto tryptone, 0.5% NaCl, 0.5% glycerol) with 0.2% glucose buffered with Tris (pH 7.2) and then resuspended in 50 ml of modified TG medium containing 20 mM added P_i (KH₂PO₄) (17–19).

Measurement of *N. punctiforme* growth by quantification of Chl-*a*. Extraction of chlorophyll *a* (Chl-*a*) was performed as described previously (20). One-milliliter aliquots were removed from each *N. punctiforme* culture into sterile 1.5-ml microcentrifuge tubes, and 90% of the medium removed after centrifugation (13,000 × *g* for 1 min). Methanol (100%) was added, and samples vortexed. The absorbance at 665 nm was measured using a BMG Labtech POLARstar OMEGA spectrometer against a 90% methanol blank. Based on an extinction coefficient of 78.74 liter/gram/cm for Chl-*a* in 90% methanol, the average Chl-*a* concentrations over 14 days were obtained by multiplying the absorbance at 665 nm by the coefficient 12.7, derived from (1/78.74) × 10³ (from the calculation of Meeks and Castenholz) (20). The calculated average Chl-*a* concentrations over 14 days were plotted against the corresponding phosphate regimes.

Effects of exposure to different phosphate concentrations on *N. punctiforme* growth. *Nostoc punctiforme* strains were cultured in Allen and Arnon medium with added P_i (KH₂PO₄) concentrations of 0, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, and 12.8 mM (14, 20). The levels of free P_i in all medium solutions were predicted using MINEQL+ software (version 4.6). The results from the MINEQL+ analyses indicated an average reduction of 50% for free P_i at pH 7.8 in Allen and Arnon medium (21, 22). Approximately 200 mg of pelleted (2,500 × *g* for 10 min) *N. punctiforme* cells were suspended in sterilized 100-ml conical flasks containing 25 ml of sterile Allen and Arnon medium containing the prescribed P_i levels. To determine the alteration in cell numbers between treatments over 14 days, 1 ml of culture was removed and pelleted, and Chl-*a* was measured as previously described (20).

Identification of putative phosphate transporters in *N. punctiforme*. To identify potential phosphate transporters in *N. punctiforme*, putative P_i transporters from *E. coli* were used as bait for Basic Local Alignment Search Tool (BLAST) searching. The search parameters were restricted to the genome sequence of *N. punctiforme* PCC 73120 (<http://img.jgi.doe.gov/cgi-bin/w/main.cgi>). The cutoff limit for retrieving sequences from BLAST results was set to a minimum E value of 1 × 10^{−5}.

Multiple sequence alignments and the percentage of identity between aligned sequences were produced for each of the retrieved putative P_i transporters in *N. punctiforme* and the established P_i transporters of *E. coli*, *B. subtilis*, *C. watsonii*, and *Synechocystis* sp. strain PCC 6803 using CLUSTAL X-2.0.10 (23). The predicted protein secondary structures and transmembrane domains (TMDs) for the identified P_i transporters in *N. punctiforme* were determined using the combinatorial TOPCONS TMD prediction algorithms (24, 25).

RNA isolation, purification, and cDNA synthesis. RNA extractions were performed using the Qiagen RNeasy plant minikit and a previously described modified protocol (26–28). The extracted RNA was purified using an Ambion DNA-free kit (Life Technologies Australia) following the manufacturer's instructions. Synthesis of cDNA from the RNA obtained was achieved using MultiScribe reverse transcriptase as described by the manufacturer's protocol (Applied Biosystems, USA).

Quantification of *N. punctiforme* gene expression levels using qPCR. Quantitative real-time PCR (qPCR) was used to quantify changes in the putative phosphate transport gene expression levels. Primers were designed to target conserved regions of predicted phosphate transporters of *N. punctiforme*, obtained through BLAST analysis, using the Primer Express program (version 2.0 for Windows 2000; Applied Biosystems) (Table 2). A pair of previously described primers designed to amplify the 16S rRNA was used to standardize the expression levels across all qPCR experiments (Table 2) (28). The primer binding efficiencies were optimized using 10, 20, 40, and 80 ng of template cDNA as the control, and cycle time of amplification was analyzed against a log scale. qPCR was performed as described in the manufacturer's protocol, using SYBR green master mix (Applied Biosystems), 20 ng of cDNA template, and 0.3 µM forward and reverse primers. All qPCR analysis was performed using the Applied Biosystems 7500 real-time PCR system and 7500 SDS software (Applied Biosystems).

Extraction of *N. punctiforme* genomic DNA. Genomic DNA was extracted from *N. punctiforme* using the Wizard genomic DNA purification kit (Promega). Extractions were performed using 100 mg (wet weight) of pelleted cells and following the manufacturer's protocol for Gram-negative bacteria. The quality and quantity of genomic DNA extracted were analyzed via electrophoresis through ethidium bromide-stained agarose gels and spectrophotometry (A_{260/280}).

Construction of insertional mutagenesis vectors for the deletion of *pstB1* in *N. punctiforme*. In order to generate the *N. punctiforme* *pstB* knockout constructs pGEM-T Easy_[pstB1::kan] and pRL-271_[pstB1::kan], primers were designed to amplify the *N. punctiforme* *pstB* gene plus ~1,200 bp up- and downstream. The amplified *pstB* product was then purified, adenylated with *Taq* DNA polymerase (Promega), and cloned into the pGEM-T Easy vector (Promega) according to the manufacturer's protocol. An additional primer pair was designed to amplify the kanamycin

TABLE 2 Primers used for gene amplification, qRT-PCR experiments, and plasmid construction^a

Primer	Sequence (5'–3')
T7	TAATACGACTCACTATAGG
Sp6	GATTTAGGTGACACTATAG
<i>pstB1</i> EXP Forward	CCTCTAGAATGGCTACCAATATTAGCAC
<i>pstB1</i> EXP Reverse	GGGATCCTTATCCAAATCTGCCGCTAA
Knockout <i>pstB1</i> Forward	AGTCAAGCGACTGTAGCTTAG
Knockout <i>pstB1</i> Reverse	GGAACGGCACTACCAACAGG
<i>pstB1</i> Kan Forward	GCAACGTTCAACTCAGCAAAAGTTCGAT
<i>pstB1</i> kan Reverse	GCCAAAGTTCTCAAAATCTCTGATGTT
<i>pstB1</i> qPCR Forward	CCAAACCCCTTTCCCAATC
<i>pstB1</i> qPCR Reverse	CGCAAACTCCGTTCTACTAATTCA
<i>pstB2</i> qPCR Forward	GATCCATAACTTGCCTCTG
<i>pstB2</i> qPCR Reverse	AGAATCTACGGGATTAGT
<i>pstB3</i> qPCR Forward	ACTCCTTCATAGCTTACAGTCTG
<i>pstB3</i> qPCR Reverse	GTGCGAGGGTCTAGTGGA
<i>pstB4</i> qPCR Forward	CTGCTTAGAACTGAAGCAGC
<i>pstB4</i> qPCR Reverse	TTCCGCGACGTTTGCCATACTC

^a Primers were used for the amplification of *N. punctiforme* *pstB1* (Npun_F0894), qRT-PCR experiments, and creation of the pGEM-T Easy_[pstB1::kan], pRL-271_[pstB1::kan], pSCR202_[pstB1], and pET15b_[pstB1] plasmids.

cin resistance (Km^r) cassette from *N. punctiforme*, with the AclI restriction sites added to the 5' and 3' ends of the primers (Table 2).

Purified Km^r cassette inserts and pGEM-T Easy_[pstB1] plasmids were then digested at the AclI restriction enzyme site according to the manufacturer's instructions (New England BioLabs), gel purified as described above, and subcloned into the pGEM-T Easy_[pstB1] construct at the corresponding unique restriction site using standard cloning procedures. The final mutagenesis vector pGEM-T Easy_[pstB1::kan] was verified by sequencing using the AB3730xl sequencing platform (Australian Genome Research Facility Ltd., Parkville, Victoria, Australia) prior to transformation into *N. punctiforme*.

Transformation of *N. punctiforme*. The *pstB1* insertional mutagenesis plasmids were transformed into electrocompetent *N. punctiforme* as previously described (26, 27, 29) with the following modifications. Approximately 10 g (wet weight) of late-exponential-phase WT *N. punctiforme* cells were harvested from stock culture via centrifugation (1,500 × g for 10 min) and washed twice with freshly made BG11. The pellets containing *N. punctiforme* cells were then resuspended in 25 ml of BG11 and sonicated on ice at 30% power for 30-s bursts with 2 min of recovery time. This process was repeated until at least 90% of filaments appeared as individual cells when visualized by microscopy. The *pstB1* mutagenesis plasmids were transformed into electrocompetent *N. punctiforme* as previously described (26, 27, 29).

Confirmation of site-specific mutagenesis of *N. punctiforme*. The insertion of the Km^r cassette within the *pstB1* gene fragment via homologous recombination was confirmed by PCR. The resulting PCR fragments were gel electrophoresed, purified, and sequenced. Single colonies were selected from the transformed cells that had been plated onto 0.5% agar and 25 µg/ml kanamycin, and site-specific double recombinants screened as previously described (26, 27, 30, 31).

Construction of *N. punctiforme* PstB1 overexpression vectors. For PstB1 overexpression (OE) constructs, oligonucleotide primers (Table 2) were designed to bind up- and downstream from the *pstB1* gene. The unique restriction enzyme sites for XbaI and BamHI were added to the 5' and 3' ends of the primers, respectively. The *pstB1* gene fragment was amplified from purified *N. punctiforme* genomic DNA. The purified *pstB1* fragment was double digested using BamHI and XbaI according to the manufacturer's protocol (New England BioLabs) (Table 2). The digested fragments were separated using agarose gel electrophoresis, and the appropriate fragments were isolated and purified using the QIAquick gel extraction kit according to the manufacturer's protocol (Qiagen).

The pSCR202 and pET15b plasmids were digested with restriction enzymes (BamHI and XbaI) to generate sticky ends using standard protocols. The linearized plasmids and the purified *pstB1* gene fragments were ligated with T4 DNA ligase at 4°C overnight. The plasmid constructs were then sequence verified to be sure the cloned genes were in frame and free of mutations.

The pSCR202_[pstB1] and pET15b_[pstB1] plasmids were transformed into *N. punctiforme* and *E. coli* BL21 electrocompetent cells, respectively, and the expression of the *pstB1* product confirmed as follows. Starter cultures of *E. coli* BL21 containing either empty pET15b vector or pET15b_[pstB1] were cultured overnight, and then 1-ml amounts were used to inoculate 100 ml of experimental cultures and the cultures incubated at 37°C for 3 h with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) according to the manufacturer's protocol (Novagen). Harvested cell pellets were mixed into 100 µl of 1× phosphate-buffered saline (PBS) and resuspended in 100 µl of 4× SDS sample buffer. Cell suspensions were sonicated according to the manufacturer's protocol (Novagen). The expression of the *pstB1* gene product (PstB) was visualized by running the total cell protein fractions through SDS-polyacrylamide gels that were stained using Coomassie blue (16). This process was also performed for *N. punctiforme* cells harboring the pSCR202_[pstB1] plasmid cultured in Allen and Arnon medium. After 72 h, cell pellets were collected (from 10 ml of culture, ~100 mg [wet weight] of cells), and protein extracted and visualized as mentioned above.

Phosphate uptake from medium by *N. punctiforme*. Triplicates of 1-ml cell cultures containing 50 mg of cells were grown in 0 mM (P_i starved), 0.8 mM (optimum or normal P_i level), and 12.8 mM (excess P_i level) P_i for 3 days. After 3 days, all samples were centrifuged at 5,000 × g for 5 min and the phosphate levels in the supernatant analyzed using the EnzChek phosphate assay kit (catalog number E6646; Thermo Fisher Scientific). A standard curve for the phosphate assay was constructed using standard P_i concentrations of 0, 2, 25, 50, 100, 150, 200, and 250 µM. In order to be within the detectable range of the P_i assay, samples from the 0.8 mM and 12.8 mM treatments were diluted to ~100 µM before analysis. A phosphate standard reaction solution containing 200 µl of 2-amino-6-mercapto-7-methylpurine riboside (MESG), 50 µl of 20× reaction buffer, and 10 µl of purine nucleoside phosphorylase (PNP) was added to each treatment, and a final reaction mixture volume of 1.0 ml created using dH₂O. All the reaction mixtures were incubated at 22°C for 30 min, and 200 µl from each treatment was loaded into a 96-well microplate. The absorbance at 360 nm was measured using a spectrometer (POLARstar Omega; BMG Labtech).

Quantification of phosphate levels in culture medium and accumulated by *N. punctiforme* WT, PstB1-overexpressing, and PstB1[−] strains after 64 h. *Nostoc punctiforme* WT, PstB1-overexpressing, and PstB1[−] cells (~1,000 mg [wet weight] of each) were pelleted, washed five times in sterile ddH₂O, and then resuspended in modified Allen and Arnon solution without P_i and purged for 72 h. Triplicate cultures of 100 mg of purged cells were then resuspended in 25 ml of Allen and Arnon medium containing 0.8 mM P_i for 64 h. Cells (3 × 10⁵) from each treatment were collected and dried in a heating block at 60°C for 8 h or until the pellets weighed ~25 mg. Cell pellets were digested in nitric acid, and total P levels analyzed for each sample using inductively coupled plasma mass spectrometry (ICP-MS). For analysis of the culture medium, the supernatants from the pelleted cells were retained and nitric acid (0.5% final concentration) added, and then P levels analyzed using ICP-MS.

Radiolabeled-phosphate (³²P) sorption from medium by *N. punctiforme*. For uptake experiments, 100-mg (wet weight) amounts of *N. punctiforme* WT and mutant cells were resuspended in 25 ml of Allen and Arnon medium containing 0.8 mM added P_i with or without 1 µCi ³²P (PerkinElmer, Melbourne, Australia). Cultures were incubated in the medium, and then samples (1 ml) collected after 0, 15, 30, 60, 120, 240, and 480 min. Following sampling, cells were immediately centrifuged at 8,000 × g for 2 min, washed in 20 mM EDTA for 5 min to remove loosely bound P_i, and then centrifuged at 8,000 × g for 2 min. The resulting cell pellets, supernatants, and media from ³²P-treated cultures were analyzed for radioisotopic content (1480 automatic gamma counter; PerkinElmer). Cell pellets from control cultures were analyzed for total protein content using the Pierce bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific) (26, 27, 30–33). Phosphate uptake into cells was calculated as pmol P_i per µg protein (26, 27, 30–33). All ³²P uptake experiments were performed in triplicate.

Radiolabeled-phosphate (³²P) sorption from medium by *E. coli* overexpressing *pstB1* of *N. punctiforme*. Conical flasks containing 7 ml modified TG medium supplemented with 1 mM IPTG and 20 mM P_i with or without 1 µCi ³²P were inoculated with 7 µl of an overnight starter culture and incubated at 37°C with constant agitation until an OD₆₀₀ of 0.5 was reached. Samples (1 ml) were removed at 0, 15, 30, 60, 120, 240, and 480 min, harvested by centrifugation (2,000 × g for 5 min), washed in 20 mM EDTA to remove loosely bound P_i, and analyzed as described above for *N. punctiforme* cells. All ³²P uptake experiments were performed in triplicate.

Statistical methods. The statistical computer program SPSS was used for all statistical analysis. Probability plots were produced for all data sets to test for normal distribution. Analysis of variance (ANOVA) tests were used to determine significant differences of mean values across the multiple treatments for qPCR, growth analysis, ICP-MS, and phosphate assays of *N. punctiforme* and *E. coli*.

FIG 3 Changes in *N. punctiforme* chlorophyll *a* levels for WT, *pstB1*-overexpressing (PstB OE), and *pstB1* knockout (PstB⁻) strains cultured in different levels of added P_i. An asterisk (*) denotes a significant difference ($P < 0.05$) between strains for each treatment. A significant difference ($P < 0.05$) in the chlorophyll *a* level across treatments (P_i levels) compared to the level in cells cultured under normal (0.8 mM) conditions is denoted by a plus sign (+) for WT cells, a hash symbol (#) for *pstB1*-overexpressing (PstB OE) cells, and a triangle (Δ) for *pstB1* knockout (PstB⁻) cells. Bars and error bars show the average values and standard deviations.

RESULTS

Response of *N. punctiforme* to different phosphate treatment regimes. The growth rates for *N. punctiforme* WT, PstB-overexpressing (PstB OE), and PstB knockout (PstB⁻) strains in different levels of P_i (0, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4, and 12.8 mM) over 14 days were recorded. For each of the 14 days, the average chlorophyll *a* concentration of each treatment was measured to determine the increase in growth (20). After 14 days, the optimum concentration of P_i was determined to be 0.8 mM for WT cells (Fig. 3). The concentrations of 0 mM and 12.8 mM were shown to cause significant ($P < 0.05$) declines in the cell growth after 14 days compared to the growth of cells grown in 0.8 mM P_i (Fig. 3).

Expression analyses of the *pstB1* in *N. punctiforme* cells exposed to different phosphate treatment regimes. Quantitative real-time PCR (qPCR) was used to determine the gene expression levels of the identified *pstB1* in *N. punctiforme* cultures exposed to low, normal, and high levels of added P_i over 64 h (Fig. 4A to C). Cells were depleted of P_i for 72 h, pelleted, and then resuspended in Allen and Arnon medium containing the optimum concentration (0.8 mM, determined as described above) of added P_i. After 1 h of growth in 0.8 mM P_i, an increase in mRNA of ~20% was measured for *pstB1* in the *N. punctiforme* WT compared to the 0.8 mM basal level (nonpurged cells from stock culture with a P_i level maintained at 0.8 mM) (Fig. 4A). After 2 h at 0.8 mM P_i, the mRNA levels of the *pstB1* gene increased to ~50% above the basal level (Fig. 4A). After 8 to 64 h of growth, the *pstB1* mRNA levels were reduced by 25 to 50% in comparison to the basal levels (Fig. 4A). For *N. punctiforme* WT cells purged and cultured under growth-limiting (0 mM) P_i, the *pstB1* mRNA levels were significantly increased (20 to 70%) for up to 32 h in comparison to the basal *pstB1* mRNA levels of nonpurged cells (Fig. 4B). The mRNA levels for *pstB1* of *N. punctiforme* WT cells purged, pelleted, and resuspended in high (12.8 mM) added P_i were significantly higher than the basal levels (~15%) and then, after 2 to 64 h, were significantly below the basal levels (50 to 70% reduction) (Fig. 4C).

Expression analyses of *pstB2*, *pstB3*, and *pstB4* in *N. punctiforme* WT, PstB1 OE, and PstB1⁻ cells exposed to different phosphate treatment regimes. The variations in mRNA levels for *pstB2*, *pstB3*, and *pstB4* were also quantified using qPCR to record the responsiveness of these genes to alterations of extracellular P_i levels and their responses in relation to the deletion or overexpression of *pstB1*.

The mRNA levels for *pstB2* increased by up to 60% in comparison to the 0-h levels for WT *N. punctiforme* cells purged of P_i for 72 h, pelleted, and resuspended in 0, 0.8, or 12.8 mM P_i over 64 h (Fig. 5, 6, and 7). The mRNA levels of *pstB2* increased significantly, by as much as 150%, for PstB1⁻ mutants after 16 to 64 h in 0 mM P_i medium (Fig. 5A). For PstB OE cells cultured in 12.8 mM P_i, the *pstB2* mRNA levels after 16 and 64 h were significantly reduced (~60% lower) in comparison to the levels in WT and PstB1⁻ cells also cultured in the high-P_i medium (Fig. 7A).

In WT cells purged of P_i, pelleted and resuspended in 0.8 mM P_i, the *pstB3* mRNA levels increased by up to 450% over 64 h in comparison to the 0-h levels (Fig. 6B). In WT cells starved of P_i over 64 h, the mRNA levels for *pstB3* increased by as much as 160% in comparison to the levels in WT cells in 0.8 mM P_i (Fig. 6B). In PstB1⁻ cells starved of P_i over 32 h, the *pstB3* mRNA levels were up to 30% lower than the *pstB3* levels in WT cells (Fig. 6B). After 64 h, the *pstB3* levels were the same for both PstB1⁻ and WT cells (Fig. 5B). For WT cells cultured in high (12.8 mM) added P_i for 64 h, the mRNA levels for *pstB3* remained similar to the 0-h levels (Fig. 6B). For PstB1 OE cells cultured in high added P_i, the *pstB3* mRNA levels were 40% lower than the *pstB3* levels in WT cells cultured in 12.8 mM P_i (Fig. 5B).

The mRNA levels of *pstB4* altered by ~40% over 64 h in WT cells cultured with normal (0.8 mM) added P_i (Fig. 6C). In WT cells starved of P_i for 64 h, the *pstB4* mRNA levels were within 20% of the 0-h levels (Fig. 6C). In PstB1⁻ cells starved of P_i for 64 h, the *pstB4* mRNA levels increased by as much as 80% in comparison

FIG 4 Alterations in mRNA levels for *N. punctiforme* *pstB1* in cells exposed to 0 (A), 0.8 (B), and 12.8 (C) mM P_i . An asterisk (*) denotes a significant difference ($P < 0.5$) between the basal (control; cells maintained at the normal P_i concentration of 0.8 mM) level and the level in cells depleted of P_i for 72 h, pelleted, and then resuspended in Allen and Arnon medium with normal (0.8 mM) added P_i for 64 h. Bars and error bars show the average values and standard deviations.

to the WT levels after 8 h and remained 15% higher after 64 h (Fig. 6C).

Accumulation of phosphates from culture medium by *N. punctiforme* WT, PstB1 OE, and PstB1[−] cells. Colorimetric assays (EnzChek phosphate assay kit) were used to determine the P_i levels in Allen and Arnon culture medium, which initially contained 0.8 mM added P_i . The phosphate levels in the culture medium after 64 h postinoculation with P_i -starved *N. punctiforme* WT and PstB1[−] cells were reduced by 11% in comparison to the initial P_i concentration in the medium (Fig. 8). The PstB1 OE strain significantly reduced the P_i levels in the medium, by 20%, in comparison with the initial concentration in the medium (Fig. 8).

Total phosphorus accumulation by *N. punctiforme* WT, PstB1 OE, and PstB1[−] cells. Inductively coupled plasma mass spectroscopy (ICP-MS) was used to quantify the total phosphorus levels in the culture medium and in *N. punctiforme* WT, PstB1 OE, and PstB1[−] cells after 64 h in Allen and Arnon medium containing 0.8 mM added P_i . The phosphorus levels in the culture medium 64 h postinoculation with *N. punctiforme* WT, PstB1 OE, and PstB1[−] cells were 186, 259, and 171 μ M less, respectively, than the levels in the uninoculated medium (Fig. 9A). The culture medium inoculated with PstB1 OE cells contained $\sim$ 80 μ M less phosphorus than the culture medium inoculated with the WT or PstB[−] strain (Fig. 9A). For *N. punctiforme* WT, PstB1 OE, and PstB1[−] cells purged, pelleted, and resuspended in 0.8 mM added P_i , the cellular accumulation of phosphorus over 64 h was measured using ICP-MS. The total cellular phosphorus levels for the WT, PstB1 OE, and PstB[−] strains were 5.6, 6.7, and 4.9 μ M, respectively (Fig. 9B).

Accumulation of radiolabeled phosphate (^{32}P) from culture and total cellular sorption by *N. punctiforme* WT, PstB1 OE, and PstB1[−] cells. Radiolabeled P_i (^{32}P) was used to measure the P_i

levels accumulated by *N. punctiforme* WT, PstB1 OE, and PstB1[−] cells over 480 min in Allen and Arnon medium containing 0.8 mM added P_i . *Nostoc punctiforme* WT, PstB-overexpressing, and PstB[−] cells were purged, pelleted, and resuspended in 0.8 mM added P_i and 1 μ Ci ^{32}P . The cellular P_i level was significantly higher (0.02 pM per μ g protein) in the PstB1 OE strain than in the WT and PstB1[−] strains after 480 min (Fig. 10A). The functional capacity of the PstB1 of *N. punctiforme* to complement the PstB of *E. coli* was tested using previously generated *E. coli* PstB[−] mutants (15). The *E. coli* PstB[−] strain complemented with PstB1 of *N. punctiforme* accumulated significantly more P_i than did the *E. coli* PstB[−] mutant (0.01 pM P_i per μ g protein more) and the PstB[−] mutant harboring the empty pET15b vector (0.02 pM P_i per μ g protein more) after 240 to 480 min of growth in modified TG medium containing 20 mM added P_i and 1 μ Ci ^{32}P (Fig. 10B). After 480 min, the *E. coli* WT strain contained significantly more P_i than both the PstB1-complemented *E. coli* mutant (0.03 pM per μ g of protein more) and the uncomplemented mutant (0.05 pM per μ g of protein more) (Fig. 10B).

DISCUSSION

The Pst P_i transport system is encoded by several genes in the *pho* regulon (Fig. 1) and is shown to be essential for the regulation of the intracellular P_i concentration. In particular, the *pho* regulon acts when cells are in low- P_i environments and when the expression of the *pst* system is induced (7). The Pst system is typically comprised of one periplasmic substrate binding protein (PstS) and three membrane-bound components (PstC, PstA, and PstB). PstB is located on the cytoplasmic side of the membrane and is the ATP-hydrolyzing subunit that provides the energy necessary to drive P_i through the membrane channel formed by PstA and PstC

FIG 5 Alterations in mRNA levels for *N. punctiforme* *pstB2* (A), *pstB3* (B), and *pstB4* (C) in *N. punctiforme* WT, *pstB1*-overexpressing (PstB1 OE), and *pstB1* knockout (PstB1⁻) cells exposed to 0 mM P_i. An asterisk (*) denotes a statistically significant difference ($P < 0.05$) between the relative mRNA levels of the *pstB1*-overexpressing and *pstB1* knockout strains in comparison to the WT. A significant difference ($P < 0.05$) between relative mRNA levels for the three strains is denoted by a hash symbol (#). Symbols and error bars show the average values and standard deviations.

(7). In *E. coli*, the deletion of *pstB* resulted in mutants with a phenotype observed to have reduced intracellular P_i accumulation (Fig. 10B) and reduced growth (see Fig. S2 in the supplemental material). In *N. punctiforme*, the deletion of *pstB1* reduced cell growth under optimum conditions (added-P_i range of 0.4 to 1.6 mM) compared to the growth of WT cultures (Fig. 3). The overexpression of *pstB1* resulted in a significant increase in cellular growth for cultures exposed to 0.2 mM added P_i, a concentration considered to be limiting for *N. punctiforme* growth. In cultures with no added P_i, the overexpression of *pstB1* showed no extra capacity to maintain cell growth compared to that of the WT. This may be due to a limitation in the ability of PstB1 to enhance P_i internalization under moderately low P_i levels or to the cells utilizing P_i from intracellular stores. It is established that cyanobacteria are highly adept at thriving in low-nutrient environments, including low P_i (nmol⁻¹ levels), by having more complex molecular uptake mechanisms than most prokaryotes (4, 12, 34). Cyanobacteria also have intracellular features, such as polyphosphate granules, that enable them to take advantage of periods of high nutrient availability (35).

***pstB1* mRNA levels increase in cells cultured under low-phosphate conditions.** The expression profile of *pstB1* in cells starved of P_i

and then resuspended in medium with low, normal, or high P_i levels showed a trend of increased expression until the intracellular levels were increased to an appropriate level needed for cell growth (Fig. 4A to C). When P_i was in excess, the *pstB1* mRNA levels were significantly reduced after 2 to 64 h. The mRNA levels of *pstB2*, *pstB3*, and *pstB4* overall showed similar trends in WT cells grown at a low P_i concentration, as their expression increased, and under high P_i levels, the mRNA levels were reduced (Fig. 5, 6, and 7). These results suggest that the Pho system is inducible in response to the environmental P_i concentration. Previous analyses of *pho* regulon genes in *Synechocystis* sp. strain PCC 6803 revealed that under low-P_i conditions, *pho* regulon (including the *pst* system components) transcript levels increase, and under conditions of excessive P_i, the *pho* regulon transcript levels are reduced (12).

In the case of the low (0 mM)-added-P_i treatment, after 64 h, the mRNA levels reverted to around basal levels. This may be indicative of reductions in overall cellular metabolism and/or the increase in other mechanisms to provide necessary P_i. To survive under environmental conditions of limiting or fluctuating P_i, some bacterial species, including *N. punctiforme*, accumulate a number of vital nutrients in the form of functionally inert polymers, including polyphosphate granules (35).

FIG 6 Alterations in mRNA levels for *N. punctiforme* *pstB2* (A), *pstB3* (B), and *pstB4* (C) in *N. punctiforme* WT, *pstB1*-overexpressing (PstB1 OE), and *pstB1* knockout (PstB1⁻) cells exposed to 0.8 mM P_i. An asterisk (*) denotes a statistically significant difference ($P < 0.05$) between the relative mRNA levels of the *pstB1*-overexpressing and *pstB1* knockout strains in comparison to the WT. A significant difference ($P < 0.05$) between relative mRNA levels for the three strains is denoted by a hash symbol (#). Symbols and error bars show the average values and standard deviations.

Manipulation of PstB1 in *N. punctiforme* alters the levels of phosphate and total phosphorus accumulated by cells. The ability of *N. punctiforme* WT, PstB1⁻, or PstB1 OE cells to accumulate P_i and total phosphorus after being purged of P_i for 72 h and then resuspended in Allen and Arnon medium containing 0.8 mM P_i was measured. For P_i accumulation from medium, only the PstB1 OE cells were shown to significantly reduce the P_i levels in the culture medium (Fig. 8). For total phosphorus, measured using ICP-MS, there were significant reductions across all culture media in comparison to the level in uninoculated medium, indicating sorption of phosphorus by cells (Fig. 9A). The PstB1 OE cells were found to significantly reduce the total phosphorus levels in the culture medium, beyond the levels measured for the WT and PstB1⁻ cells. These findings indicate that while the overexpression of PstB1 did not increase cellular growth, its increased activity increased the capacity of cells to take up extracellular P_i.

The total cellular accumulation of phosphorus by WT, PstB1⁻, and PstB1 OE cells was measured using ICP-MS. The WT and PstB1⁻ cells contained similar amounts of total accumulated phosphorus, whereas the PstB1 OE cells accumulated significantly more phospho-

rus over 64 h (Fig. 9B). This suggests that the overexpression of *pstB1* in *N. punctiforme* increases P_i internalization through the Pst systems. There are limited studies showing *in vivo* accumulation of P_i in cells with *pstB* deletion or overexpression. *In vitro* kinetic experiments have characterized PstB as having a nucleotide-binding site and shown that mutations of residues within this site cause reduced P_i transport through the Pst system and the constitutive synthesis of alkaline phosphatase (36, 37).

The accumulation of radiolabeled P_i (³²P) from culture medium by the WT, PstB1⁻, and PstB1 OE strains over 480 min was also measured, and the PstB1 OE cells were found to have accumulated significantly more P_i after 480 min (Fig. 10A). Interestingly, no strong phenotype was observed for the PstB1⁻ mutant in regard to *in vivo* detection of total phosphorus or P_i uptake. This lack of a strong phenotype may be attributable to PstB2, PstB3, and/or PstB4 compensating for the loss of PstB1 with regard to P_i accumulation. Previously, the deletion of single metal transporters or metal transport system components in *N. punctiforme* was not shown to result in altered phenotypes, and this was attributed to the duplication and redundancy of other transport mechanisms which could compensate for the

FIG 7 Alterations in mRNA levels for *N. punctiforme* *pstB2* (A), *pstB3* (B), and *pstB4* (C) in *N. punctiforme* WT, *pstB1*-overexpressing (PstB1 OE), and *pstB1* knockout (PstB1⁻) cells exposed to 12.8 mM P_i. An asterisk (*) denotes a statistically significant difference ($P < 0.05$) between the relative mRNA levels of the *pstB1*-overexpressing and *pstB1* knockout strains in comparison to the WT. A significant difference ($P < 0.05$) between relative mRNA levels for the three strains is denoted by a hash symbol (#). Symbols and error bars show the average values and standard deviations.

loss (26, 27, 31). In *Mycobacterium smegmatis*, PstS⁻ mutants showed reduced growth under low-P_i conditions; however, the P_i levels were shown by *in vivo* ³²P accumulation studies not to alter, due to compensation by alternative P_i transport systems (38).

***pstB2*, *pstB3*, and *pstB4* mRNA levels altered to compensate for the loss or increase in PstB1 in *N. punctiforme* PstB1⁻ and PstB OE mutants.** To further investigate potential compensation for the loss of PstB1 by the other *pstB* genes in *N. punctiforme*, we

FIG 8 Colorimetric assay-based quantification of phosphate levels in Allen and Arnon medium (0.8 mM added P_i) 64 h after inoculation of medium with *N. punctiforme* cells that had been depleted of P_i for 72 h prior. An asterisk (*) denotes a statistically significant difference ($P < 0.05$) between the levels in uninoculated medium and medium inoculated with *N. punctiforme* *pstB1*-overexpressing (PstB1 OE) cells. Bars and error bars show the average values and standard deviations.

FIG 9 Total phosphorus levels in culture medium and accumulated by *N. punctiforme* cells 64 h after inoculation as quantified using ICP-MS. (A) Quantification of total phosphorus levels removed from medium by *N. punctiforme* WT, *pstB1*-overexpressing (PstB1 OE), and *pstB1* knockout (PstB1⁻) cells. (B) Quantification of phosphorus levels accumulated by *N. punctiforme* WT, *pstB1*-overexpressing (PstB1 OE), and *pstB1* knockout (PstB1⁻) cells after 64 h of growth in Allen and Arnon medium with 0.8 mM added phosphate. An asterisk (*) denotes a statistically significant difference ($P < 0.05$) between the levels in uninoculated medium and medium inoculated with an *N. punctiforme* strain. A significant difference ($P < 0.05$) between the levels in WT and *pstB1*-overexpressing cells is denoted by a hash symbol (#). Bars and error bars show the average values and standard deviations.

measured the RNA levels of *pstB2*, *pstB3*, and *pstB4* in WT, PstB1⁻, and PstB1 OE cells cultured in low, medium, or high levels of added P_i (Fig. 5, 6, and 7). The PstB1⁻ mutants had increased mRNA levels of *pstB2*, *pstB3*, and *pstB4*, particularly in the case of PstB1⁻ cells cultured in low P_i levels. While there is an obvious redundancy across the three Pst systems, it may also safeguard *N. punctiforme* cells from death by maintaining P_i accumulation under environmental conditions of low P_i. In *Synechocystis* sp. strain PCC 6803, two Pst systems exist (Pst1 and Pst2), with signaling between the two systems occurring under stressful low-P_i conditions; the capacity to compensate for the loss of either system has not been shown (12).

PstB1 complements the loss of PstB in *E. coli*. The accumulation of radiolabeled P_i by *E. coli* PstB⁻ mutants and *E. coli* mutants complemented with the PstB1 of *N. punctiforme* demonstrated that *N. punctiforme* PstB1 can complement function in *E. coli* with regard to increasing the cellular P_i levels. However, the intracellular P_i accumulation by the *E. coli* mutant complemented with the PstB1 of *N. punctiforme* was lower than that in the WT, suggesting that *N. punctiforme* PstB1, although functional in *E. coli*, is less efficient than the native homolog (Fig. 10B). Previous complementation studies of individual *pst* operon components have also demonstrated that the complementation of a single

gene, such as *pstA* or *pstB*, does not restore P_i uptake to the level recorded in the WT. This is due to the *pst* operon being transcribed as a single transcript and not as individual genes (39, 40).

The incomplete complementation by PstB1 of *N. punctiforme* in *E. coli* may be attributed to the ~40% difference in peptide identity between the two sequences. The sequence alignments of the four PstB peptides from *N. punctiforme*, as well as PstB peptide sequences from *B. subtilis*, *C. watsonii*, and *Synechocystis* sp. strain PCC 6803 and the PstB of *E. coli*, revealed that amino acids 46 to 52 are conserved across this very diverse group of prokaryotes, reiterating that this region is of importance for the proper functioning of PstB (see Fig. S1 in the supplemental material). This conserved region has previously been determined as a GXX GXG Lys-Ser/Thr nucleotide-binding sequence, and mutations of individual residues in this motif have been shown to reduce P_i internalization in *E. coli* (36, 37, 41, 42). The residues in this motif are essential for energy coupling and are therefore required for the correct functioning of PstB and the Pst system (43). Histidines (H) have also been determined to be a key P_i-binding residue for proteins (44). Throughout the PstB sequences of *N. punctiforme*, *E. coli*, *B. subtilis*, *Synechocystis* sp. strain PCC 6803, and *C. watsonii*, there are multiple histidine residues; however, the only one that is conserved is within a conserved motif (VTHNM) between residues

FIG 10 Levels of radiolabeled phosphate (^{32}P) accumulated by *N. punctiforme* and *E. coli* strains. (A) Values for *Nostoc punctiforme* WT, *pstB1*-overexpressing (PstB1 OE), and *pstB1* knockout (PstB $^{-}$) cells. (B) Values for *Escherichia coli* WT, *pstB* knockout (PstB $^{-}$), PstB $^{-}$ harboring pET15b empty vector (PstB $^{-}$ pET15b), and PstB knockout mutants complemented with PstB1 of *N. punctiforme* (Npun PstB comp). A statistically significant difference ($P < 0.05$) between the values for PstB1-complemented, PstB $^{-}$ pET15b empty vector control and PstB $^{-}$ *E. coli* strains is denoted by double asterisks (**). A statistically significant difference ($P < 0.05$) between the values for PstB1-complemented *E. coli* PstB $^{-}$ mutants and WT cells is denoted by a hash symbol (#). Symbols and error bars show the average values and standard deviations.

216 and 220 (see Fig. S1). Differences in histidine content may also be indicative of P_i -binding affinities for the different PstBs; however, this requires further exploration.

Its role in P_i accumulation and the results of complementation analyses in *N. punctiforme* in this study show that the PstB1 of *N. punctiforme* is a functional component of its extensive P_i transport systems and is involved in increasing the levels of internalized P_i . The redundancy of the P_i transport system in *N. punctiforme* suggests that it can compensate for the loss of PstB1 through altering the regulation of the other three PstB system components.

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